Epicubenol Synthase and the Stereochemistry of the Enzymatic Cyclization of Farnesyl and Nerolidyl Diphosphate

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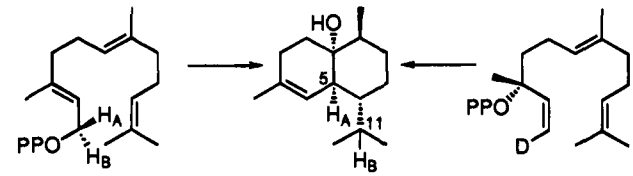
(+)-Epicubenol (**1**) is a sesquiterpene alcohol belonging to the cadinane family which has been isolated from a variety of soil organisms, including *Streptomyces* sp. LL-B7.[1] The enantiomeric (−)-epicubenol has also been isolated from cubeb oil as well as from extracts of several other plants.[2] More than 20 years ago, Arigoni described the results of extensive incorporation experiments carried out with intact plants and microorganisms and proposed a stereochemical model for the biosynthesis of a group of biogenetically related cadinane, picrotoxane, and sativane sesquiterpenes, based on the cyclization of the universal acyclic sesquiterpene precursor, farnesyl diphosphate (FPP, **2**), and the postulated intermediacy of its tertiary allylic isomer nerolidyl diphosphate (NPP, **3**).[3] In the intervening years, there have been no tests of this model at the enzyme level. Recently we described the preparation of a cell-free extract of *Streptomyces* sp. LL-B7 which catalyzed the cyclization of FPP to (+)-epicubenol (**1**).[4] Using specifically deuterated samples of FPP, we documented the operation of both 1,3- and 1,2-hydride shifts in the formation of (+)-epicubenol and presented evidence for a cyclization mechanism consistent with the intermediacy of nerolidyl diphosphate.[4,5] We now describe experiments with (+)-epicubenol synthase which confirm the proposed intermediacy of (3*R*)-nerolidyl diphosphate in the cyclization reaction and which establish the stereochemical course of the conversion of farnesyl diphosphate to (+)-**1**.

Preparative scale incubation of (1*R*)-[1-^{2}H]FPP[6] (**2a**) (5 μM), containing [1-^{3}H]FPP as an internal standard (final sp act. 23.8 nCi/μmol), was carried out for 2 h at 30 °C using crude epicubenol synthase[4] obtained from an 8-L culture of *Streptomyces* sp. LL-B7 to give 150 nmol of (+)-epicubenol. After addition of 3 mg of unlabeled synthetic (±)-epicubenol[7] as carrier, the resulting products were rigorously purified by flash column chromatography (SiO_2, 15:85 ether/pentane) and analyzed by 61.42 MHz ^{2}H NMR spectroscopy in $CHCl_3$. The purified epicubenol (**1a**) displayed a single peak at δ 1.69 ppm, corresponding to deuterium at C-5[4] (Scheme 1). The corresponding ^{2}H NMR spectrum of (+)-epicubenol (**1b**) (120 nmol) derived from an analogous incubation of (1*S*)-[1-^{2}H]FPP[6] (**2b**) displayed a single peak at δ 1.95 ppm in $CHCl_3$, corresponding to deuterium at C-11.[4]

To test the role of nerolidyl diphosphate in the cyclization, partially purified epicubenol synthase[8] (4 mL) was incubated for 2 h at 30 °C with (3*R*)-(1*Z*)-[1-^{3}H]NPP[9] (**3**) (5 μM, 71.7 mCi/mmol, 1.43 μCi) in the presence of 30 mM $MgCl_2$ and 30 mM Na_2MoO_4[10] (Scheme 2). The incubation was halted by addition of 2 mL of 1 M Na_2EDTA and the mixture extracted with ether. The resulting epicubenol was treated with a 5-fold excess of OsO_4 in pyridine for 12 h at room temperature.[4] The derived triol **4** (13.6 nCi, 1.0% conversion) was readily separated by flash column chromatography (SiO_2, 80:20 ether/pentane) from the corresponding heptols derived from nerolidol and farnesol that had been generated by competing phosphatase- and Mg^{2+}-catalyzed hydrolysis[11] of the substrate, NPP. Analogous incubation of (3*S*)-(1*Z*)-[1-^{3}H]NPP (5 μM, 65.1 mCi/mmol, 1.3 μCi) and OsO_4 treatment, on the other hand, failed to give labeled triol (<0.03% conversion), thus demonstrating that epicubenol synthase is specific for (3*R*)-NPP.

Scheme 1

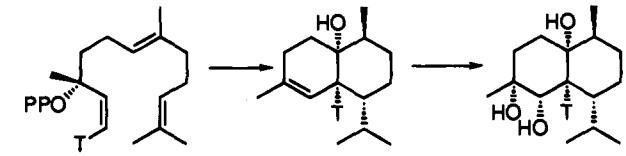


2a H_A=D, H_B=H
2b H_A=H, H_B=D
2c H_A=H_B=D

1a H_A=D, H_B=H
1b H_A=H, H_B=D
1c H_A=H_B=D

3a

Scheme 2



3 **1** **4**

The stereochemistry of the cyclization of (3*R*)-NPP to (+)-epicubenol was established by incubation of (3*R*)-(1*Z*)-[1-^{2}H]-NPP and analysis of the deuterium distribution in the derived epicubenol by GC/selected ion mass spectroscopy. Reference samples of [5-^{2}H]epicubenol (**1a**), [11-^{2}H]epicubenol (**1b**), and [5,11-^{2}H$_2$]epicubenol (**1c**), prepared by cyclization of (1*R*)-[1-^{2}H]FPP (**2a**), (1*S*)-[1-^{2}H]FPP (**2b**), and [1,1-^{2}H$_2$]FPP (**2c**), respectively, were used to assign unambiguously the *m/z* 204 [M − H_2O], 179 [M − C_3H_7 (*i*-Pr)], and 161 [M − H_2O − *i*-Pr] fragments in the electron impact spectrum of **1** (Scheme 3). Although the parent M^+ peak (*m/z* 222) was not observed for any sample, the presence of deuterium at C-11 in **1b** was easily recognized by comparison of the ratios of deuterated (*m/z* 205) to nondeuterated (*m/z* 204) species in the [M − H_2O] fragment with the corresponding ratios of the *m/z* 179/180 and 161/162 pairs which both result from loss of the deuterated *i*-Pr (C_3H_6D) moiety (Table 1). By contrast, the deuterium content of the [M − H_2O] and [M − *i*-Pr] fragments was essentially identical when deuterium was present at C-5 of **1a**, indicating that dehydration of the parent ion must involve predominant loss of the H-9 and/or H-1 protons.[12]

With the MS fragmentation pattern of epicubenol assigned, incubation of (3*R*)-(1*Z*)-[1-^{2}H]NPP (**3a**) (5 μM, 334 nmol), containing (3*R*)-(1*Z*)-[1-^{3}H]NPP (final sp act. 68.6 nCi/μmol, 92% d_1),[13] was carried out with partially purified epicubenol

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(8) Epicubenol synthase was purified 10-fold by ion exchange chromatography of the crude enzyme on DE-52. The resulting preparation (1.7 mg of protein/mL) had a specific activity of 0.21 nmol h^{-1}/(mg of protein)$^{-1}$. Most importantly, the residual phosphatase activity was reduced to 0.3–0.5 that of the observed cyclase activity.
(9) Cane, D. E.; Ha, H.-J. *J. Am. Chem. Soc.* **1986**, *108*, 3097–3099.
(10) Na_2MoO_4 was used to inhibit the phosphatase activity. Cf.: Croteau, R.; Cane, D. E. In *Methods in Enzymology. Steroids and Isoprenoids*; Law, J. H., Rilling, H. C., Eds; Academic Press: New York, 1985; Vol. 110, pp 383–405.
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(12) The slight increase in the proportion of nondeuterated species in the *m/z* 179/180 and 161/162 clusters derived from **1a** may indicate a secondary deuterium isotope effect discriminating against the loss of the isopropyl side chain from the deuterated parent when deuterium is at C-5.

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Scheme 3

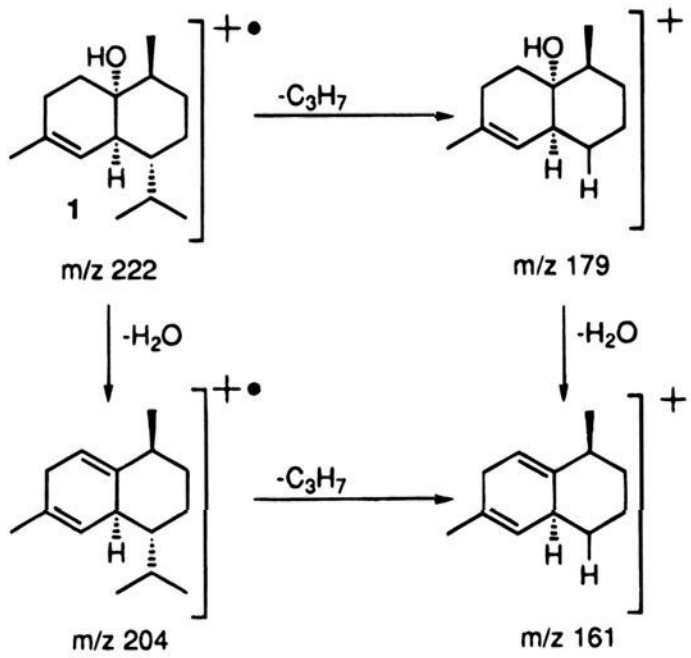


Table 1. GC/Selected Ion Mass Spectrometric Analysis of (+)-Epicubenol (**1**) Derived from (1*R*)-[1-^{2}H]FPP (**2a**), (1*S*)-[1-^{2}H]FPP (**2b**), [1,1-$^2H_2$]FPP (**2c**), and (3*R*)-(1*Z*)-[1-^{2}H]NPP (**3a**)

	percent			
m/z	**1a**	**1b**	**1c**	**1a**[a]
206			87	
205	83	93	13	55 (86)[b]
204	17	7		45 (14)[b]
180	80	6	86	51 (80)[b]
179	20	94	14	49 (20)[b]
162	77	8	84	52 (81)[b]
161	23	92	16	48 (19)[b]

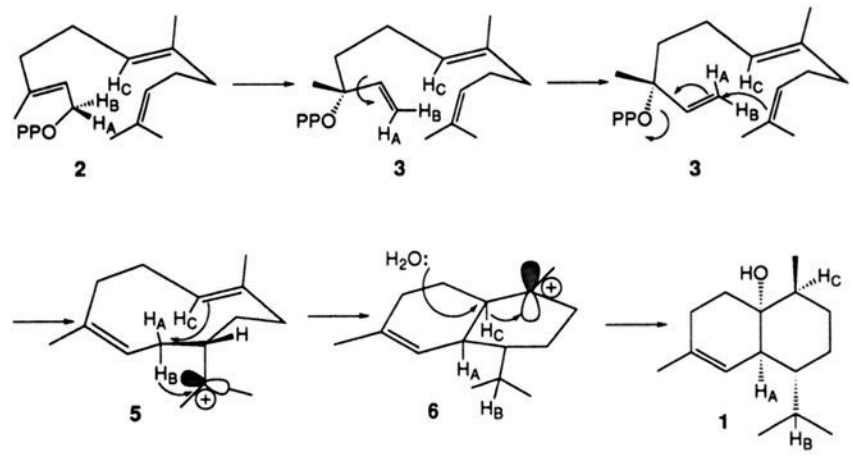
[a] Derived from (3*R*)-(1*Z*)-[1-^{2}H]NPP. [b] After correction for the 36% d_0 species in the parent epicubenol **1a**.

synthase (65 mL) in the presence of $MgCl_2$ (30 mM) and Na_2MoO_4 (30 mM) for 2 h at 30 °C. The epicubenol derived from **3a** was 64% d_1 as established by GC−MS analysis and measurement of the [M − CH_3] peaks in the spectrum of the corresponding TMS ether generated by treatment of a portion of the labeled epicubenol with TMS-imidazole. The observed depletion in deuterium relative to the [1-^{2}H]NPP substrate probably reflects, at least in part, the operation of a secondary deuterium isotope effect on the isomerization and/or cyclization step catalyzed by epicubenol synthase.[14] The remainder of the epicubenol thus obtained (12.8 nmol) was subjected to GC/selected ion MS analysis. After correction for the 36% epicubenol-d_0 in the sample, the deuterium distribution was found to be essentially identical to that found in epicubenol (**1a**) derived from (1*R*)-[1-^{2}H]FPP (**2a**) (Table 1), thereby establishing unambiguously the presence of deuterium exclusively at C-5 in **1a** obtained from (3*R*)-(1*Z*)-[1-^{2}H]NPP (Scheme 1).

Since cyclization of both(1*R*)-[1-^{2}H]FPP (**2a**) and (3*R*)-(1*Z*)-[1-^{2}H]NPP (**3a**) gives [5-^{2}H]epicubenol (**1a**), it can be concluded that the initial isomerization of FPP to (3*R*)-NPP takes place

(13) To determine the ^{2}H content of the NPP sample, **3a** was hydrolyzed with alkaline phosphatase to (3*R*)-(1*Z*)-[1-^{2}H]nerolidol, which was converted to its TMS ether derivative by treatment with (trimethylsilyl)imidazole. GC-MS analysis of the nerolidol TMS ether showed that the substrate **3a** contained 92% deuterium, based on the relative intensity of the [M − CH_3] ions at *m/z* 279 and 280.

Scheme 4



with suprafacial stereochemistry (Scheme 4), consistent with the demonstrated stereochemistry of the isomerization steps catalyzed by the sesquiterpene cyclase trichodiene synthase[15] and the mechanistically related FPP-NPP isomerase,[16] as well as the established suprafacial stereochemistry of the analogous isomerization of geranyl diphosphate to linalyl diphosphate in the formation of numerous cyclic monoterpenes.[17] Ionization of (3*R*)-NPP and attack by the 10−11 double bond on the *re* face of the cisoid allylic cation−pyrophosphate anion pair, corresponding to net anti stereochemistry in the allylic substitution reaction, will generate the *cis*-germacradienyl cation **5**.[18] In **5** the original H-1 *si* hydrogen of FPP (H_B) can properly align with the vacant p orbital of the neighboring 2-propyl cation side chain, consistent with the observed stereochemistry of the 1,3-hydride shift. A second electrophilic cyclization gives the cadinanyl cation **6**, which upon a 1,2-hydride shift and syn capture of water generates epicubenol (**1**). These results directly confirm the earlier proposals of Arigoni for the intermediacy of NPP in the formation of related cadinane, picrotoxane, and sativane sesquiterpenes[3] and fully support previously developed stereochemical models of sesquiterpene biosynthesis.[18]

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(14) On the basis of the measured 3.8% conversion of NPP to epicubenol, the isotope effect can be estimated to have an upper limit of 1.4, using the formula $^D(V/K) = \log(1 - f)/[\log(1 - fR/R_0)]$ where f = fractional conversion to product, R_0 = isotopic abundance in the substrate, and R = isotopic abundance in the product. (Cleland, W. W. *Crit. Rev. Biochem.* **1982**, *13*, 385−428). Since the isotopic enrichment was measured for only a single percent conversion, the value of 1.4 must be considered only a very rough estimate at best and is likely to be high based on known α-deuterium isotope effects on solvolytic reactions (Melander, L.; Saunders, W. H. *Reaction Rates of Isotopic Molecules*; John Wiley & Sons: New York, 1980; pp 172−173.) Nonetheless, secondary deuterium isotope effects on *V/K* for terpenoid cyclizations have previously been reported (Croteau, R.; Wheeler, C. J.; Cane, D. E.; Ebert, R.; Ha, H.-J. *Biochemistry* **1987**, *26*, 5383−5389) and support the notion that ionization of the allylic diphosphate substrate may be the rate-limiting chemical step in such reactions.

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(18) All S_N' displacements of allylic diphosphate esters that have been investigated have been found to take place with the same net anti stereochemistry. For reviews, see: Cane, D. E. *Chem. Rev.* **1990**, *9*, 1089−1103. Cane, D. E. *Tetrahedron* **1980**, *36*, 1109−1159. Cane, D. E. *Acc. Chem. Res.* **1985**, *18*, 220−226. Croteau, ref 17.